**Transcriptomic Mapping of the 5-HT Receptor Landscape**

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# Abstract

# Introduction

# Results

**Transcriptomic overview of 5-HT receptors landscape**

We analysed the single-cell scRNA-seq dataset provided by the Allen Institute {Yao, 2023 #2828} focusing on the expression of Htrs RNA across approximately 4 million brain cells. The scRNA-seq dataset comprehensively encompassed all known 14 Htr subtypes. 65.84% of cells expressed RNA of at least one Htr. Prevalence of Htrs across the entire dataset was considerably different ranging from 0.09% of Htr3b to 34.26% of Htr1f (Figure 1a). RNA of 6 Htr was found in less than 2.5% of the cells (Htr1d, Htr2b, Htr3a, Htr3b, Htr5b, Htr6). On the other hand, RNA of Htr1f, Htr2a and Htr2c was present in at least 1 every 5 cells. Average amount of RNA expression also varied across receptors (Supplementary Figure 1a). Interestingly, the variation in amount of RNA shared ≈half of the variability with the prevalence, i.e., receptors found in more cells also tended to be expressed more at the single cell dimension. Beside the prevalence and amount of expression, also the distribution was considerably different. This is exemplified by looking at the distribution of the Htr1 and Htr2 families across different cell type neighborhoods (Figure 1b). Neighborhoods were defined both by location and neurotransmitter (Supplementary Figure 1b, Table 1). When looking at the UMAP distributions for individual Htr, considerable differences were present also within family (Supplementary Figure 2). We analyzed this differences more in detail by grouping cells by neurotransmitter, neighborhoods or class. These categorizarions divided cells in a higlhy skewed manner (Supplementary Figure 1c), for example when looking at groups by neurotransmitter release, 3 groups (Glut, Gaba and unassigned) made up for almost the totality of cells (98.47%). Expectedly, the vast majority of cells was classified as excitatory (Glut) (50.79%) and ≈1 every 5 cells was found to release GABA (20.62%). All the other neurotransmitter were found in less than 1% of the cells, in particular, 5-HT releasing neurons (Sero) were found in only 0.04% of the cells. Pattern of Htrs expression across different neurotransmitter groups exhibited a relatevely high Pearson correlation coefficient (r=0.6±0.03). Sero and cholinergic neurons (Chol) showed the most distinct patterns of expression with respectively r=0.32±0.04 and r=0.370.05. To evaluate the uniqueness of Htrs RNA expression per group we employed a Random Forest Classifier aiming at decoding the grouping variable from the Htrs expression (Figure 1c). Overall accuracy of the model was 36.66%. Reflecting the correlation analysis, Sero and Chol were among the groups with higher true positive (TP) rate (Sero=75.34%, Chol=42.01%). Cells not expressing any Htr were also identified succesfully (no-Htr=80.69%). Noradrenaline (Nora) and glycine (GABA-Glyc) releasing neurons were also identified almost half of the times (Nora=38.71% and GABA-Glyc=48.96%). To understand the contribution of each Htr in each prediction we calculated the mean absolute SHAP values for each receptor and neurotransmitter. The shap values associated with the mean prevalence enable us to understand which are the defining features of each grop. Here we can see, for example, that the identification of Sero neurons is determined mainly by expression of Htr1a and Chol neurons by Htr4 and Htr5b. Absence of expression can also contribute to the classification, e.g., Htr4 is rarely expressed by Nora neurons. When looking at different neighborhoods the accuracy of the model was 51.82%. The model could differentiate best the NN-IMN-GC, TH-EPI-Glut and Pallium-Glut groups (NN-IMN-GC=74.17%, TH-EPI-Glut=66.05% and Pallium-Glut=57.2%, Supplementary Figure 3a). NN-IMN-GC includes all the cells not releasing any neurotransmitter, their classification is thereofre predictably strongly influenced by absence of any Htr. On the other hand, TH-EPI-Glut cells were characterized by high expression of Htr7 and low expression of Htr2a and Htr4. Across classes, differences in Htrs expression were even more evident (Figure 1d). 7 groups could be identified with a TP rate > 40%: 04 DG-IMN Glut, 05 OB-IMN GABA, 09 CNU-LGE GABA, 18 TH Glut, 22 MB-HB Sero, 25 Pineal Glut, and 32 OEC (Supplementary Figure 3b). 04 DG-IMN Glut were charachterized by high expression of Htr4 and absence of, the usually prevalent, Htr2c. Similarly, 05 OB-IMN GABA cells showed virtual absence of Htr2c but expression of Htr1f; 09 CNU-LGE GABA cells show high Htr1b and low Htr7; 17 MH-LH Glut exhibited high levels of Htr5b and Htr4; 18 TH Glut showed high levels of Htr7 and virtual absence of Htr4; 22 MB-HB Sero, miroring the results showed by Sero neurons, were charachterized by Htr1a; at last, 34 Immune cells were identified by absence of any Htr expression. Correlation between Htrs expression across the totality of cells ranged from -0.03 (Htr1f-Htr3a) to 0.311 (Htr4-Htr2c). Considerable correlation was also found for the Htr7-Htr2c (r=0.264) and Htr1f-Htr2a (r=0.212) pairs (Figure 1e). Interestingly, correlation patterns were not stable across neighboroods (Supplementary Figure 4a). For example, Pallium-Glut exhibited a negative correlation between Htr4-Htr2a not evident from the entire dataset. Of note, TH-EPI-Glut showed the highest absolute correlation across all neighboroods with r=0.609 between Htr5b-Htr4 and a unique negative correlation between Htr5b-Htr7 and Htr4-Htr7. To investigate the underlying cause of the correlations we investigated colocalization between Htrs using the same stringent threshold used by the original authors of the dataset {Yao, 2023 #2828}. Across the entire dataset we observed that the most expressed genes, Htr1f and Htr2c, were often colocalized with other genes (Figure 1g). This was a driving factor for correlation. After dividing the dataset across neighboroods we noticed that Pallium-Glut and TH-EPI-Glut were the most peculiar groups. they showed the lowest Htr2c colocalization, i.e., cells expressing any other Htr colocalized with Htr2c <20%, in all other groups Htr2c colocalization was at least 40%. Moreover, Pallium-Glut was the only group showing a uniquely low Htr7 colocalization (14.77%) on the other hand, TH-EPI-Glut showed the highest (63.86%). Only rarely a cell was found to express uniquely one Htr, 86.41±1.69% of cells indeed expressed at least 2 Htrs (Figure 1f). Surprisingly, 22.88±1.9% of cells expressed at least 5 Htrs. The extensive expression across different Htr classes and the considerable coexpression within cells point at the complexity of the 5-HT system even at the single cell dimension. To facilitate an understanding of the downstream cellular effects of 5-HT we aggregated receptors according to their main intracellular effector. We aggregated Htr1 and Htr5 due to their inhibitory effect (cAMP decrerase), Htr4, Htr6 and Htr7 because of the shared downstream effect of increasing cAMP. Htr2 is the only one that causes an Ca2+ increase while Htr3 is the only ionotropic receptor. For each cell we determined the the principal pathway activated by 5-HT by looking at the amount of RNA of each Htr. Afterwards we divided the cells across the different neighborhoods (Figure 1h). Ht3 were present only in a small minority of cortical inhibitory neurons.In the telencephalon, the absolute majority of both Pallium-Glut and Subpallium-Gaba cells were linked to Htr1/5, ≈one quarter of cells instead featured Htr2 as primary effector. Subcortical cells exhibited a more balanced proportion without any absolute majority and a considerable presence of Htr4/6/7.. In the following sections we will take a deeper look at Htrs grouped by effector and we will take advantage of the spatial information provided by the MERFISH dataset of {Zhang, 2023 #2887} regarding 9 Htrs.

**Htr1 & Htr5**

Receptors belonging to these two families have an inhibitory effect on the host cell, they are coupled to Gᵢ and cause a downstream decrease of cAMP and activation of GIRK channels {Sharp, 2020 #2888; McCorvy, 2015 #2889}. Hr1a RNA have a stable prevalence of ≈10% in the brain in the RNA-seq dataset (excluding non-neuronal cells and immature neurons), with virtual absence in the TH-EPI-Glut group (Figure 2a). Htr1a co-localized most frequently with Htr1f, Htr2c and Htr2a (Figure 2b) and only in a minority of cases was expressed alone (<10%). Expression across classes was highly correlated between the RNA-seq and MERFISH datasets (Figure 2a) and show an almost perfect proportional relationship. Highest expression was found in 5-HT neurons of the mid- and hindbrain (class 22 MB-HB Sero, Figure 2c), nonetheless, cortical excitatory neurons (01 IT-ET Glut) had the higher absolute number of cells expressing Ht1a. To pinpoint the spatial location we first identified the clusters highly enriched with Htr1a RNA with a threshold of 70%, i.e., to be classified as enriched at least 70% of cells must express the receptor. Only 6.52% of Htr1a expressing cells were contained in enriched clusters, pointing at a relatively low importance in the clustering algorithm. Looking at the spatial distribution across divisions, the highest prevalence was found in cortical region of the pallidum (PAL) and hippocampus (HPF) (Figure 2d). At a more granular level, the highest prevalence was observed in the dorsal raphe (DR). DR expression is reflection of the high prevalence in Sero neurons outlined above, DR contains a substantial proportion of Sero neurons. The hippocampal structure exhibiting the higher prevalence was medial entorhinal cortex (ENTm) while the medial septum nucleus (MS) and the diagonal band nucleus (NDB), two structures linked to generation of theta waves {Winson, 1978 #2908}, contributed substantially to the expression in PAL. Levels of of expression were stable across the anterior-posterior axis (Figure 2e-f). Htr1b exhibited a more diverse pattern of expression across neighboroods (Figure 3a) ranging from 10 to 30%. Highest prevalence was observed in the MB-HB-Glut-Sero-Dopa group, glutamatergic, serotonergic and dopaminergic neurons located in midbrain and hindbrain. Colocalization showed a similar pattern compared to Htr1a (Figure 3b) and also here only a minority of cells expressed Htr1b alone (<10%).Looking at expression across classes, 09 CNU-LGE GABA class showed the highest prevalence (58.06%) closely followed by 22 MB-HB Sero (53.73%) (Figure 3c). High expression in 09 CNU-LGE GABA was in sharp contrast with Htr1a that showed only minimal expression in this class (1.61%). Also in this case 01 IT-ET Glut exhibited the highest absolute number of Htr1b expessing cells.17.48% of Htr1b expressing cells belonged to highly enriched clusters andstriatum (STR) showed by far the highest prevalence with >60% (Figure 3d-e-f). Surprisongly, Caudoputamen (CP) showed a prevalence of >40%. Nucleus accumbens (ACB), olfactory tubercle (OT), lateral septal nucleus (LSc) and the parabigeminal nucleus (PBG) all exhibited a prevalence of >30%. Htr1d was expressed at a much lower level, never exceeding 7% prevalence in any neighborhood (Supplementary Figure 5a). It colocalized at highest levels with Htr2c, Htr1f and Htr1b (Supplementary Figure 5b) and only rarely was expressed alone (<5%). Similarly to Htr1b, expression was highest in 09 CNU-LGE GABA and 22 MB-HB Sero (Supplementary Figure 5c). Notably, 09 CNU-LGE GABA exhibited the highest absolute number of cells surpassing 01 IT-ET Glut. Only a small minority of cells belonged to enriched clusters (2.08%). The paraventricular nucleus of the thalamus (PT and PVT) showed the highest prevalence at >4% (Supplementary Figure 5d-e-f). Htr1f showed the highest expression of all 5-HT receptors in the RNA-seq dataset. Higher prevalence was found in the Pallium and Subpallium groups (Figure 4a), reaching ≈50%. Other groups showed a prevalence of 30-40% with TH-EPI-Glut at ≈20% (Figure 4a). Htr1f was found to colocalize the most with Htr2a and Htr2c (Figure 4b). In 30% of cases Htr1f was the only Htr expressed in a cell, colocalization decreased linearly with the number of coexpressed Htrs (Figure 4b). Notably, the slope of the linear regression between values provided by RNA-seq and MERFISH was significantly lower (Figure 4c). The two datasets are still highly correlated, with 66% of shared variability. Htr1f was broadly expressed across almost all classes, including some non-neuronal cells, cells belonging to the pineal gland being an exception. In absolute numbers, Cortical glutamatergic cells showed the highest expression. Spatial distribution showed a peculiarly asymettric pattern with expression concentrated in the most anterior regions. Highest expression was observed in STR, olfactory areas (OLF) and the cortical subplate (CTXsp)reaching over 20% (Figure 4d-e-f). Specifically, highest expression was observed in nucleus accumbens (ACB) and olfactory tract (OT), similarly to Htr1b. The accessory olfactory bulb (AOB) was the OLF structure with the highest prevalence. Claustrum (CLA), on the other hand, was the CTXsp structure exhibiting the highest prevalence.Both Htr5a and Htr5b were not included in the MERFISH dataset, therefore we do not have any direct spatial visualization of their expression. Htr5a was expressed at 8-16% prevalence across all neighborhoods (Supplementary Figure 6a) and colocalized the most with Htr1f, Htr2c and Htr2a (Supplementary Figure 6b). Expression was broadly distributed across many classes, altough only at lower levels compared to other Htrs (Supplementary Figure 6c). Only one cluster was considered enriched with Htr5a in the entire RNA-seq dataset, 3453 PAG-PPN Pax5 Sox21 Gaba. This cluster was located mainly in the midbarin reticular nucleus (RR, Supplementary Figure 6d-e).Htr5b was expressed at a much lower level (Supplementary Figure 7a), with a maximum of ≈%5 in TH-EPI-Glut. Interstingly, this receptor was expressed at considerable levels only in the 17 MH-LH Glut class (≈50% prevalence). This was caused by high levels of expression in the medial habenula (MH, Supplementary Figure 7d-e), a structure involved in the response to stress and fear {Chou, 2016 #2913;Soria-Gomez, 2015 #2910;Winson, 1978 #2908;Yamaguchi, 2013 #2909}. Some expression was also evident in the posterior part of the brain, specifically in the inferior olivary complex (IO), a structure strongly linked to cerbellar Purkinje cells {Loyola, 2023 #2914}. This expression was driven by a single supertype, 253 IO Fgl2 Glut.

**Htr2**

The Htr2 family is mainly linked to Gq/11 and causes excitation by increasing intracellular Ca2+. Htr2a, famous for being instrumental in mediating the effects of psychedelics {Nichols, 2016 #854}, is found across the brain with highest prevalence in cortical groups, Pallium-Glut and Subpallium-GABA (Figure 5a). Colocalization was highest with Htr1f and Htr2c (Figure 5b). Considerable expression (≈40%) was found in 01 IT-ET Glut, 07 CTX-MGE GABA and 16 HY-MM Glut classes (Figure 5c). Htr2a was also prevalent across many other classes accross the whole brain. Similarly to Htr1f, also here the MERFISH dataset hinted at a lower overall expression when compared to RNA-seq. Shared variability between the two was, nonetheless very high. CTXsp showed the highest prevalence, reaching more >≈12% (Figure 5d). Isocortex and STR exhibited both ≈5% prevalence. At a structure level, surprisingly, two structures belonging to the mammillary complex (dorsal premammillary nucleus, PMd and tuberomammillary nucleus,TMd) were in the top ten. The mammillary complex has been linked to Alzheimer´s disease {Huang, 2023 #2915}, and memory {Roy, 2017 #2916}. CLA and the endopiriform nucleus (EPd) showed the highest absolute prevalence. INterestingly, CLA has been proposed to play an important role in mediating the effects of psychedelic compounds {Doss, 2022 #2917}. Expression of Htr2a was highest in frontal regions of the brain, decaying linearly to vistua absence in the cerebellum (Figure 5e-f). Htr2b was found only in a minority of neurons and was not included in the MERFISH dataset. NO cluster was found to be enriched with Htr2b. Interestigly, neurons belonging to the Pineal Glut class showed the highest prevalence at 7.34% (Supplementary Figure 8c). Htr2c was found at highest prevalence in the MB-HB-Glut-Sero-Dopa and Hy-EA-Glut-Gaba groups (Figure 6a). Interestingly, groups with higher prevalence showed also higher levels of expression. Colocalization was highest with Htr1f, Htr4 and Htr7. In similar fashion to Htr2a, also here there were discrepancies between the RNA-seq and MERFISH methods (Figure 6c). Expression was broadly distributed across many different classes, also subcortically. The majority of cells expressing Htr2c belonged to enriched clusters (Figure 6d-e-f). Highest prevalence was found in STR. Similarly to Htr1b, ACB, CP and OT exhibited the highest prevalence.

**Htr4, Htr6 and Htr7**

These receptor are all connected to Gs {McCorvy, 2015 #2889}, leading to increasing cellular levels of cAMP and excitation. Htr4, similarly to Htr2c, showed highest prevalence (>40%) in the MB-HB-Glut-Sero-Dopa and Hy-EA-Glut-Gaba groups (Figure 7a). It colocalized the most with Htr2c and Htr1f (Figure 8b). Discrepancies in amount of expression between RNA-seq and MERFISH were present also here (Figure 7c). This did not affect notably, however, the correlation between the two datasets. Expression across classes did not exhibited any peculiar pattern. Spatial distribution,however, was more interesting, exhibiting a peculiarly high prevalence in one specific structure of the STR, OT. PAL and HPF also exhibited relatively considerable prevalence (≈10%). Dentate gyrus (DG) granule cells were the main driver of the high prevalence. We do not have MERFISH information about the rarely expressed Htr6and no enriched cluster was present in the RNA-seq dataset (Supplementry Figure 11). On the other habd, Htr7 was expressed in more than ≈10% of cells. It reached 60 % in the TH-EPI Glut group, and considerable amounts (≈40%) in MB, HB and HY groups (Figure 8a). Colocalization was the highest with Htr2c and Htr1f (Figure 8b). Expression was broadly distributed across classes present in HY, MB and TH (Figure 8c). This was reflected in the MERFISH dataset, showing highest prevalence in HY and TH (Figure 8d). At a structure level, the parafascicular nucleus of TH (PF) showed the highest prevalence (>30%).

# Discussion

# Materials and Methods

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**Htr1a**

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# Data and materials availability

All the code used to process the dataset is available at https://github.com/RobertoDF/De-Filippo-et-al-2022, pre-computed data structures can be downloaded at 10.6084/m9.figshare.20209913. All figures and text can be reproduced using code present in this repository, each number present in the text is directly linked to a python data structure. The original dataset is provided by the Allen Institute and available at https://allensdk.readthedocs.io/en/latest/visual\_coding\_neuropixels.html.

# Figures

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# Supplementary Figures

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